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Antigenic variation in *Plasmodium knowlesi* malaria: Identification of the variant antigen on infected erythrocytes

(antibody-mediated agglutination/cloned parasites/immunoprecipitation/lactoperoxidase-catalyzed radioiodination)

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ABSTRACT Erythrocytes infected with mature asexual stages of *Plasmodium knowlesi* express a new surface antigen such that rhesus monkey antisera specifically agglutinate these cells. Cloned parasites can express different antigenic variants of this antigen. The variant antigen has been identified by comparison of the surface membrane antigens of a clone and of an antigenic variant of that clone of different agglutination phenotype. After lactoperoxidase labeling, ^{125}I -labeled proteins of M_r s 210,000 and 190,000 with one clone and of M_r s 205,000–200,000 with the antigenic variant were only immunoprecipitated by antisera containing homologous anti-variant antibody. Antigens of the same M_r from each clone were labeled by [^{35}S]methionine incorporation during parasite growth and also specifically immunoprecipitated only by agglutinating antisera. Therefore, the variant antigens are malarial proteins rather than modified host proteins.

New antigens on the surface of malaria-infected erythrocytes (RBC) are potential targets for immune responses that could destroy the intraerythrocytic asexual parasite. In the case of rhesus monkey RBC infected by schizonts of *Plasmodium knowlesi*, there is evidence that the parasite has evolved the capacity to vary the antigenicity of a new surface antigen and thereby evade variant-specific immunity. These schizont-infected RBC (SI-RBC) are specifically agglutinated by antisera from infected animals (1). However, the antigen responsible for antibody-mediated agglutination varies during chronic infection (2) or upon reinfection of monkeys with the same variant (3). Cloned parasites also can be induced to undergo antigenic variation *in vivo* in the presence of homologous agglutinating antibody (4). The antibody-mediated agglutination assay, which defines the variant antigen phenotype of different parasite populations, is called the schizont-infected-cell-agglutination (SICA) test (3). The nature of the erythrocyte membrane component (SICA antigen) responsible for antigenic variation in *P. knowlesi* has not been described, nor is it known whether this antigen is synthesized by the malaria parasite or an altered host component.

Because different sera specifically agglutinate SI-RBC of different SICA phenotypes, we established the following criteria to identify individual variant antigens. The variant antigen should be immunoprecipitated from SI-RBC of a particular phenotype only by antisera capable of agglutinating those cells. Furthermore, any particular SICA antigen should not be immunoprecipitated from uninfected erythrocytes nor from infected erythrocytes bearing another SICA antigen as defined by a different agglutination specificity. To implement these criteria, we isolated two *P. knowlesi* clones of different SICA phenotype, Pk1(A+) and Pk1(B+)1+. Because Pk1(B+)1+ was an antigenic variant of Pk1(A+) (4) induced by agglutinating antibody,

any differences in antigenic composition between these clones would most probably relate to the expression of different variant antigens on the cell surface. Here we identify antigenically and structurally distinct protein antigens from each clone that are only immunoprecipitated by agglutinating antisera. These variant proteins are shown to be synthesized by the malaria parasite.

MATERIALS AND METHODS

Cloned Parasites. Clone Pk1(A+) was produced by micro-manipulation and expansion in a naive rhesus monkey (*Macaca mulatta*) of a single SI-RBC infected with *P. knowlesi* of the Malaysian-H strain (4). Clone Pk1(B+)1+ was derived by antigenic variation *in vivo* from Pk1(A+) and recloning. An animal was infected with clone Pk1(A+), drug cured, and reinfected with Pk1(A+). The parasites that appeared [Pk1(B+)] were of a different variant antigen phenotype to Pk1(A+). Pk1(B+)1+ was derived by recloning from Pk1(B+). Parasitized cells infected with immature (ring-stage) parasites were collected from naive monkeys infected with these clones and cryopreserved. SI-RBC of each SICA phenotype were obtained after thawing and 20–24 hr *in vitro* culture (4).

Antisera. Antisera were tested for antibody-mediated agglutination of SI-RBC by the SICA-test (1, 2) as described elsewhere (5). Monkeys were infected with cloned parasites, drug cured, and subsequently immunized with SI-RBC of the same clone in Freund's incomplete adjuvant (3) to yield high-titer agglutinating antisera specific for the immunogen (i.e., antisera 2–7 of Table 1). Rabbits also were immunized with SI-RBC of each clone to produce variant-specific agglutinating antisera (i.e., antisera 18 and 19 of Table 1). Crossreactive antisera that agglutinated both clones were from monkeys chronically infected with many SICA variants of the same strain (i.e., antisera 9 and 10 of Table 1). Several antisera that do not agglutinate these clones provided negative controls (Table 1): antisera 11, 12, and 15 were from animals infected or immunized with nonagglutinable (i.e., SICA[–]) parasites (5); and antisera 13 and 14 were from animals infected with different strains.

Lactoperoxidase-Catalyzed Radioiodination. SI-RBC consisting of >95% parasitized RBC were purified by density gradient centrifugation and radioiodinated by the lactoperoxidase/ $\text{Na}^{125}\text{I}/\text{H}_2\text{O}_2$ method (7) as described (8) under conditions of minimal intracellular labeling.

Immunoprecipitation. Radioiodinated SI-RBC were washed in phosphate-buffered saline, and 1×10^9 cells were resuspended in 3.5 ml of phosphate-buffered saline. A cocktail of protease inhibitors (100 μl) was added to give final concentrations (in 4.0 ml) of 1 mM EGTA, 1 mM EDTA, 1 mM *p*-chloromercuribenzoate, 1 mM 1,10-phenanthroline, 2 mM phenyl-

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Abbreviations: SICA, schizont-infected cell agglutination; RBC, erythrocyte(s); SI-RBC, schizont-infected erythrocyte(s).

Table 1. Reciprocal agglutination titers of rhesus monkey and rabbit antisera and a monoclonal antibody with SI-RBC of cloned parasites

No.	Antiserum or antibody		SI-RBC	
	Donor*	Immunogen†	Pk1(A+)	Pk1(B+)1+
1	Normal M	None	<10	<10
2	M A25A	Pk1(A+)	40,960	<10
3	M 398D		20,480	<10
4	M 184B		10,240	<10
5	M 189B		<10	40,960
6	M 529R	Pk1(B+)1+	<10	40,960
7	M 195E		<10	81,920
8	M 608H		<10–160‡	20,480
9	M 331	SICA[+], noncloned Malaysian H strain	5,120	20,480
10	M 332		2,560	5,120
11	M 780F	SICA[–] clone S ^c (5)	<10	<10
12	M 705D		<10	<10
13	M 125		160‡	<10
14	M 621	Noncloned Hackeri strain	<10	<10
15	M Q148B	SICA[–], noncloned Malaysian H strain	<10	<10
16	13C11§	—	<10	<10
17	Normal R	None	<10	<10
18	R 3	Pk1(A+)	10,240	<10
19	R 7	Pk1(B+)1+	<10	>20,480

* Rhesus monkey (M) and rabbits (R) are identified by animal number.

† Monkeys were infected or immunized with SI-RBC for sera 2–8, chronically infected for sera 9 and 10, infected and drug cured for sera 11–14, and immunized with purified merozoites for serum 15. Rabbits were immunized with SI-RBC for sera 18 and 19.

‡ Agglutination was undetectable or an incomplete mat of agglutinated cells.

§ This monoclonal antibody immunoprecipitates a malarial protein of M_r 230,000 from detergent extracts of SI-RBC. It binds to the surface of extracellular *P. knowlesi* merozoites, resulting in agglutination and reduced invasion (6).

methylsulfonyl fluoride, 0.2 mM *N*^ε-tosyllysine chloromethyl ketone and 0.1 mM *N*-tosylphenylalanine chloromethyl ketone. After the ingredients were mixed, 400 μ l of 10% NaDodSO₄ in phosphate-buffered saline was added; the sample was mixed and incubated for 15 min at 23°C. We had established that, whereas the variant antigens of *P. knowlesi* are extracted from SI-RBC with 1% Triton X-100, the yield is much greater if 1% NaDodSO₄ is used (unpublished data). The clear supernatant of the NaDodSO₄ extract was recovered by centrifugation (5 min, 13,000 \times g, Eppendorf centrifuge). Sufficient 30% (wt/vol) bovine serum albumin and 20% (wt/vol) Triton X-100 in phosphate-buffered saline were added to the NaDodSO₄ supernatant to give final concentrations of 1% and 2%, respectively. The antigen extract was diluted 1:1 with NETT buffer (0.15 M NaCl/5 mM EDTA/50 mM Tris chloride/1% Triton X-100, pH 7.4) containing 1% (wt/vol) bovine serum albumin and was divided into 200- to 400- μ l aliquots to which 30 μ l of serum was added. After 2–4 hr at 23°C, 150 μ l of 1:1 (vol/vol) protein A-Sepharose (Pharmacia, Uppsala, Sweden)/NETT buffer containing 1% bovine serum albumin was added, the samples were shaken 30 min, and the beads were washed as described (8). Antigen was eluted by addition of 80 μ l of NaDodSO₄ sample buffer (9) containing 5% NaDodSO₄ and heating 5 min at 100°C.

[³⁵S]Methionine Labeling of Malarial Proteins. Ring-stage parasitized cells from cryopreservation (4) were resuspended in RPMI 1640 medium containing 10% of the normal level of L-methionine plus additional D(-)-glucose (2 g/liter), 20 mM

Hepes, 0.25% NaHCO₃, 15% (wt/vol) normal rhesus monkey serum, and 25 μ g of gentamicin, 10 μ g of hypoxanthine, and 200 μ Ci of L-[³⁵S]methionine (1,440 Ci/mmol, Amersham; 1 Ci = 3.7×10^{10} Bq) per ml. The concentration of infected erythrocytes was 2×10^7 cells per ml and the hematocrit was 2–5%. The cells were incubated 19–22 hr at 37°C in sealed tissue culture flasks gassed initially with 3% O₂/6% CO₂/91% N₂. SI-RBC were harvested by density gradient centrifugation (8).

Reaction of Antibody with Intact SI-RBC. SI-RBC labeled by [³⁵S]methionine uptake were resuspended in phosphate-buffered saline at 5×10^8 cells per ml; 200- μ l aliquots were incubated with 100 μ l of rhesus monkey antiserum (30 min at 23°C) before being washed twice in 3 ml of phosphate-buffered saline containing 10% (vol/vol) bovine serum albumin (pH 7.2). The cells were resuspended in 200 μ l of phosphate-buffered saline, and 10 μ l of the cocktail of protease inhibitors was added as for extraction of radioiodinated SI-RBC. After the sample was mixed, 200 μ l of 2% (wt/vol) Triton X-100 was added, mixing was repeated, and samples were incubated 30 min on ice. Detergent-solubilized material was recovered by centrifugation (5 min, 13,000 \times g, Eppendorf centrifuge), and antigen-antibody complexes were purified by using Protein A-Sepharose.

NaDodSO₄/Polyacrylamide Gel Electrophoresis. Radiolabeled antigen extracts were electrophoresed on 5–15% gradients of acrylamide (9). Gels containing ¹²⁵I were dried after Coomassie blue staining and analyzed by autoradiography (10). Gels containing ³⁵S were stained, destained, and processed for fluorography (11) with EN³HANCE (New England Nuclear).

RESULTS

Immunoprecipitation of ¹²⁵I-Labeled Antigens (¹²⁵I-Antigens). ¹²⁵I-Antigens were extracted with NaDodSO₄ from lactoperoxidase-labeled SI-RBC and, after addition of Triton X-100, were immunoprecipitated by using a panel of antisera of defined agglutination specificity (Table 1). The results of NaDodSO₄/polyacrylamide gel electrophoresis are shown in Fig. 1 and summarized from several experiments in Table 2.

Comparison of the first lane in Fig. 1 (normal serum) and subsequent lanes (antisera from immunized animals) shows that many ¹²⁵I-antigens were specifically immunoprecipitated by anti-*P. knowlesi* antisera. When the overall patterns of labeled antigens from Pk1(A+) were examined, it was apparent that only bands of M_r s 210,000 and 190,000 showed a consistent and specific relationship to the agglutinating properties of these antisera (Fig. 1A and Table 2). Only antisera that agglutinate clone Pk1(A+) immunoprecipitated the ¹²⁵I-antigens of M_r s 210,000 and 190,000. Minor ¹²⁵I-antigens of M_r s 182,000 and 170,000 were specifically immunoprecipitated in one experiment (Table 2 and Fig. 1A). Antisera that specifically agglutinated Pk1(B+)1+ did not immunoprecipitate the M_r 210,000/190,000 doublet from Pk1(A+) (antisera 5 and 6, Fig. 1A). ¹²⁵I-Antigens of M_r s 210,000 and 190,000 were not immunoprecipitated by any antisera from ¹²⁵I labeled SI-RBC of clone Pk1(B+)1+ (Fig. 1B and Table 2).

When clone Pk1(B+)1+ was examined similarly, another doublet of ¹²⁵I-antigens, M_r s 205,000/200,000, was immunoprecipitated only by antisera that agglutinate these cells (Fig. 1B and Table 2). On some gels the specifically immunoprecipitated ¹²⁵I-antigens from Pk1(B+)1+ electrophoresed as a single band of M_r 205,000–200,000 (Table 2). The variant specific antisera that only agglutinates Pk1(A+) SI-RBC and that immunoprecipitated the M_r 210,000/190,000 doublet from Pk1(A+) did not immunoprecipitate the M_r 205,000–200,000 ¹²⁵I-antigens from Pk1(B+)1+. Antigens of M_r 205,000–200,000 were

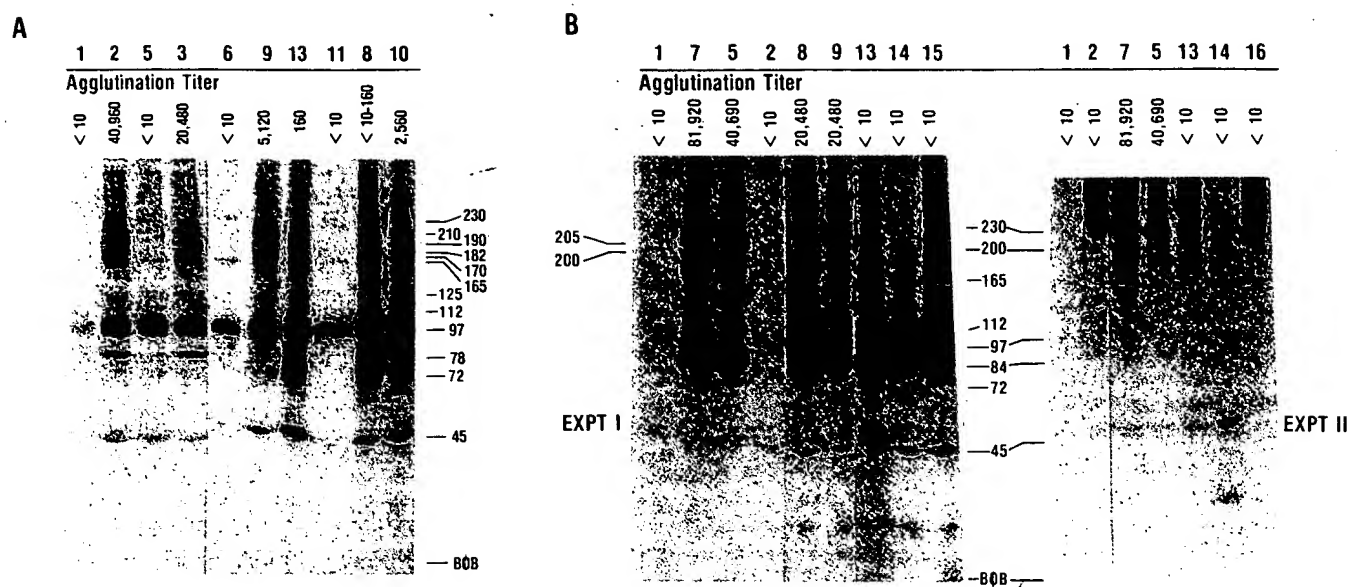


FIG. 1. Immunoprecipitation of ^{125}I -antigens from extracts of radioiodinated SI-RBC of clones Pk1(A+) (A) and Pk1(B+)1+ (B) by monkey antisera of defined agglutinability. SI-RBC were labeled by the lactoperoxidase method and extracted in 1% NaDodSO₄. The extract was made 0.5% in NaDodSO₄ and 1.5% in Triton X-100, antigens were immunoprecipitated with monkey antisera, and the complexes were purified with protein A-Sepharose; antigens were separated on a 5–15% polyacrylamide gel and detected by autoradiography. The results of two experiments are shown for Pk1(B+)1+. Each gel lane is identified by the serum number used for immunoprecipitation and its reciprocal agglutination titer with SI-RBC of the same clone (see Table 1). The M_r s of major ^{125}I -antigens are in kilodaltons. BQB, bromphenol blue dye front; EXPT, experiment.

not immunoprecipitated by any antisera from Pk1(A+) SI-RBC (Fig. 1A and Table 2). None of these antigens of Pk1(A+) or Pk1(B+)1+ were seen with labeled normal RBC.

The accumulated data in Table 2 emphasize the fact that although 15 ^{125}I -antigens were identified with *P. knowlesi* SI-RBC, only two ^{125}I -antigens from each clone showed a consistent and specific correlation in their immunoprecipitation and agglutination patterns. It also should be noted that specific immunoprecipitation of Pk1(A+) antigens of M_r s 210,000 and 190,000 and of Pk1(B+)1+ antigens of M_r 205,000–200,000 was ob-

served with each of two or three variant specific antisera in each experiment. No nonagglutinating antiserum immunoprecipitated these bands in any experiment. The results obtained with crossreactive antisera, which agglutinate both clones, confirmed that these ^{125}I -antigens are variant specific antigens (Table 2). The crossreactive antisera immunoprecipitated ^{125}I -antigens of M_r s 210,000 and 190,000 from Pk1(A+) and ^{125}I -antigens of M_r 205,000–200,000 from Pk1(B+)1+.

There was qualitative and quantitative variation in the patterns of other ^{125}I -antigens (M_r s 230,000, 165,000, 125,000,

Table 2. Accumulated data for immunoprecipitation of ^{125}I -antigens from cloned parasites by rhesus monkey sera of defined agglutinability

^{125}I -Antigen, $M_r \times 10^{-3}$	Immunoprecipitation, no. of experiments*							
	SI-RBC of clone Pk1(A+)				SI-RBC of clone Pk1(B+)1+			
	Normal serum	Specific anti-Pk1(A+)	Specific anti-Pk1(B+)1+	Crossreactive	Normal serum	Specific anti-Pk1(A+)	Specific anti-Pk1(B+)1+	Crossreactive
230	—	+(3)	+(3)	+(3)	—	+(4)	+(4)	+(4)
210	—	+(3)	—	+(3)	—	—	—	—
200–205†	—	—	—	—	—	—	+(4)	+(4)
190	—	+(3)	—	+(3)	—	—	—	—
182	—	+(1)	—	+(1)	—	—	—	—
170	—	+(1)	—	+(1)	—	—	—	—
165	—	+(3)	+(3)	+(3)	—	+(4)	+(4)	+(4)
125	—	+(3)	+(3)	+(3)	—	+(2)	+(2)	+(2)
112	—	+(2)	+(2)	+(2)	—	+(3)	+(3)	+(3)
97	+(2)	+(3)	+(3)	+(3)	+(2)	+(4)	+(4)	+(4)
84	—	—	—	—	+(1)	+(3)	+(3)	+(3)
78	+(1)	+(1)	+(1)	+(1)	+(1)	+(1)	+(1)	+(1)
72	+(1)	+(2)	+(2)	+(2)	+(1)	+(2)	+(2)	+(2)
45	+(2)	+(2)	+(2)	+(2)	+(3)	+(4)	+(4)	+(4)

The results of three experiments with Pk1(A+) and four experiments with Pk1(B+)1+ are shown. The sera are described in greater detail in Table 1: "normal serum" refers to serum no. 1; "specific anti-Pk1(A+)" refers to sera nos. 2, 3, and 4, which agglutinate only Pk1(A+); "specific anti-Pk1(B+)1+" refers to sera nos. 5, 6, and 7, which agglutinate only Pk1(B+)1+; and "crossreactive" refers to sera nos. 9 and 10, which agglutinate both clones.

* +, Detected in at least one experiment, with the number of positives in parenthesis; —, not detected.

† A doublet of ^{125}I -antigens of M_r s 205,000 and 200,000 was immunoprecipitated twice. In two other experiments, only one band of M_r 200,000 was evident.

112,000, 97,000, 84,000, 78,000, 72,000 and 45,000) precipitated by antisera from different animals with any particular antigen extract (Fig. 1 A and B). This is not unexpected in view of the complexity of the immune response with infected and immunized monkeys. Because these ^{125}I -antigens were immunoprecipitated by nonagglutinating and agglutinating antisera (Table 2), they appeared to be unrelated to antigenic variation. For example, the M_r 78,000 ^{125}I -antigen was only seen in one experiment with each clone. It was strongly immunoprecipitated from Pk1(A+) (Fig. 1A) by three agglutinating antisera (nos. 2, 3, and 10) but was not immunoprecipitated by another strong agglutinating antiserum (no. 9). The status of the M_r 84,000 ^{125}I -antigen of Pk1(B+)1+ is uncertain. It appears to be present only in this clone but was immunoprecipitated by both nonagglutinating and agglutinating antisera.

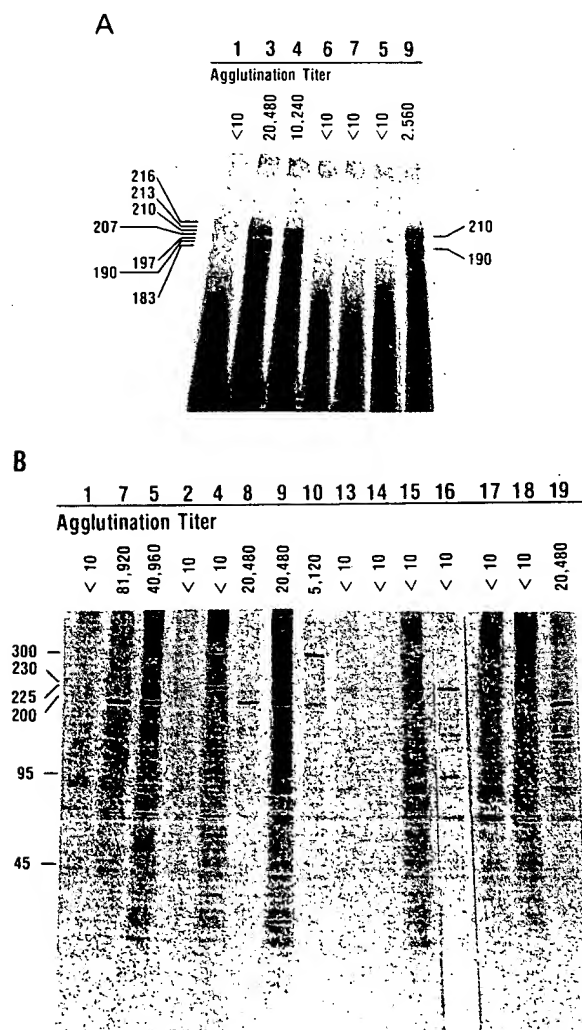


FIG. 2. Immunoprecipitation of [^{35}S]methionine-labeled antigens from SI-RBC of clones Pk1(A+) (A) and Pk1(B+)1+ (B) after reaction of antisera with intact SI-RBC. Malarial proteins were labeled by 19–22 hr of *in vitro* culture of infected RBC in the presence of [^{35}S]methionine. SI-RBC were purified by density gradient centrifugation and incubated 30 min with various antisera of defined agglutinability. After being washed to remove unbound antibody, SI-RBC were extracted with 1% Triton X-100, and antigen-antibody complexes were purified by using protein A-Sepharose. Antigens were electrophoresed on a 5–15% polyacrylamide gel and detected by fluorography. Gel lanes are identified by the number of the serum (see Table 1) used for immunoprecipitation and its reciprocal agglutination titer with SI-RBC of the same clone.

Variant-Specific Immunoprecipitation of Malarial Proteins. To determine whether the SICA antigens are synthesized by the malaria parasite, malarial proteins were radiolabeled by *in vitro* culture with [^{35}S]methionine. Under these conditions, the contribution of RBC to protein synthesis was negligible. Intact [^{35}S]methionine-labeled SI-RBC of each clone were incubated with sera, washed to remove unbound antibody, and solubilized in detergent for immunoprecipitation analysis (Fig. 2).

With Pk1(A+), a complex group of malarial proteins were immunoprecipitated only by antisera that agglutinate these SI-RBC (Fig. 2A). The two major [^{35}S]methionine-labeled parasite antigens had M_r s of 210,000 and 190,000, identical to the M_r of the ^{125}I -antigens specifically immunoprecipitated from Pk1(A+) after radioiodination (Fig. 1A). Other minor [^{35}S]methionine-labeled antigens; also specifically immunoprecipitated, had M_r s of 216,000, 213,000, 207,000, 197,000, and 183,000. Incubation of [^{35}S]methionine-labeled cells of clone Pk1(A+) with normal monkey serum or high-titer agglutinating antisera specific for Pk1(B+)1+ failed to immunoprecipitate these antigens (Fig. 2A).

Analysis of [^{35}S]methionine-labeled antigens of Pk1(B+)1+ SI-RBC (Fig. 2B) also confirmed the radioiodination experiments. A major [^{35}S]methionine-labeled parasite antigen of M_r 205,000–200,000 was immunoprecipitated only by antisera that agglutinate Pk1(B+)1+. A minor [^{35}S]methionine-labeled antigen of M_r 225,000 also showed variant-specific immunoprecipitation. Because proteins of identical M_r and immunoprecipitation specificity were observed both by surface labeling and by biosynthetic labeling, we conclude that the variant antigens are proteins of malarial origin rather than host origin.

Several additional [^{35}S]methionine-labeled antigens that were not immunoprecipitated in a variant-specific manner were identified in some experiments (Fig. 2B) but not others (Fig. 2A). Chief among them were [^{35}S]methionine-labeled antigens of M_r s 300,000 and 230,000. Although the subcellular location of the M_r 300,000 antigen is unknown, the M_r 230,000 antigen is known to be specifically immunoprecipitated by a monoclonal antibody (Fig. 2B) that reacts with the surface of merozoites and mature intraerythrocytic parasites (6). The occasional identification of [^{35}S]methionine-labeled antigens other than the variant antigen probably represents interaction of antibody with intracellular components from a small proportion of broken SI-RBC.

DISCUSSION

There have been no previous studies on the biochemical nature of variant antigens on the surface of rhesus monkey RBC infected with schizonts of *P. knowlesi*. These results demonstrate that the RBC membrane components responsible for variant-specific antibody-mediated agglutination are proteins synthesized by the malaria parasite. We compared the antigenic compositions of a clone of *P. knowlesi* and another clone derived from the first by antibody-induced antigenic variation *in vivo*. The two clones, Pk1(A+) and Pk1(B+)1+, differ in SICA or agglutination phenotype. Their variant antigen phenotypes also are distinguished by an indirect immunofluorescence test for variant-specific binding of rhesus monkey antibody, which showed that >99% of the SI-RBC of each clone are of the same variant antigen phenotype (4). The criterion used to identify the SICA variant antigen was that this molecule should be immunoprecipitated from SI-RBC of a particular SICA phenotype only by sera capable of agglutinating those cells. Our results identify protein antigens from each clone that appear to be antigenically and structurally unique to each clone and that are immunoprecipitated from each clone only by antisera that ag-

glutinate that clone. Therefore, these antigens are variant-specific antigens.

The major variant antigens of Pk1(A+) had M_r s of 210,000 and 190,000 and those of Pk1(B+)1+ had M_r s of 205,000–200,000. Minor ^{125}I -antigens or [^{35}S]methionine-labeled antigens also were specifically immunoprecipitated in some experiments (Table 2 and Fig. 2). We do not know whether the different specifically immunoprecipitated protein antigens of each clone are related to each other structurally or encoded on one or several genes. Different SICA antigens could have arisen in the expanded cloned parasites by change of variant antigen phenotype during expansion. Alternatively, the higher M_r forms of the SICA antigen may be precursors of the lower M_r forms. Finally, the higher and lower M_r forms may be due to incomplete proteolytic cleavage as a reproducible *in vitro* artifact occurring at the time of cell solubilization and immunoprecipitation, despite the addition of a cocktail of protease inhibitors.

The most compelling evidence that the variant-specific protein antigens are on the SI-RBC surface membrane derives from the origin of the parasites. These antigens were identified in a clone and, in another clone of different agglutination phenotype, derived from the first by antigenic variation in the presence of agglutinating antibody. The agglutination of SI-RBC reflects the interaction of antibody with a surface antigen on these cells. Therefore, we expect very few differences in the antigenic composition of these clones other than the expression of different variant antigens on the SI-RBC surface membrane. The agglutination properties of different antisera correlated exactly with their capacity to immunoprecipitate the variant antigens (Table 2). Secondary evidence for location of the variant antigens on the cell surface comes from the fact that the ag-

glutination specificity of different antisera correlated with the capacity of antibody to bind to [^{35}S]methionine-labeled variant antigens with intact cells (Fig. 2). Furthermore, the conditions of radioiodination that label the variant antigens predominantly label surface membrane proteins (8).

These results show that an intracellular malaria parasite can synthesize a protein antigen and transport it for expression on the RBC surface membrane. The biochemical identification of the SICA variant antigen is a first step towards examining whether other malaria parasites, including the human malarias, express variant antigens and whether they also export protein antigens to the surface of infected cells.

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1. Eaton, M. D. (1938) *J. Exp. Med.* 67, 857–869.
2. Brown, K. N. & Brown, I. N. (1965) *Nature (London)* 208, 1286–1288.
3. Brown, K. N., Brown, I. N., Trigg, P. I., Phillips, R. S. & Hills, L. A. (1970) *Exp. Parasitol.* 28, 318–338.
4. Barnwell, J. W., Howard, R. J. & Miller, L. H. (1982) *Ciba Found. Symp.* 94, 117–136.
5. Barnwell, J. W., Howard, R. J. & Miller, L. H. (1983) *J. Immunol.* 128, 224–226.
6. Epstein, N., Miller, L. H., Kaushal, D. C., Udeinya, I. J., Renner, J., Howard, R. J., Asofsky, R., Aikawa, M. & Hess, R. L. (1981) *J. Immunol.* 127, 212–217.
7. Phillips, D. R. & Morrison, M. (1971) *Biochemistry* 10, 1766–1771.
8. Howard, R. J., Barnwell, J. W., Kao, V., Daniel, W. A. & Aley, S. B. (1982) *Mol. Biochem. Parasitol.* 6, 343–367.
9. Laemmli, U. K. & Favre, M. (1973) *J. Mol. Biol.* 80, 575–599.
10. Swanson, R. & Shank, P. R. (1978) *Anal. Biochem.* 86, 184–192.
11. Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* 46, 83–88.